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17. Constant-flow air samplers fitted with charcoal tubes approved by the National Institute for Occupational Safety and Health were used to sample air in the enclosure of the treatment and true control groups.
18. We thank C. Carl and M. DeMerritt for supplying the sugar maples and poplars, M. E. Montgomery for permission to cite unpublished work, and especially D. Rhoades for support, encouragement, and good ideas. R. T. Holmes, Cap'n K, and three anonymous reviewers helped improve the manuscript. D. Archambault, K. Hoy, T. Morrill, D. Munson, and D. Ward helped with chemical analysis. M. J. Richards drafted Fig. 1. Supported by grant DEB-8022174 from the National Science Foundation (J.C.S. and R. T. Holmes) as part of our continuing studies of herbivory at the Hubbard Brook Experimental Forest.

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Cooperative Immunoassays: Ultrasensitive Assays with Mixed Monoclonal Antibodies

Abstract. *Mixtures of certain monoclonal antibodies appear to bind human chorionic gonadotropin in a "cooperative" fashion because they form circular complexes with the hormone. Experiments illustrate how this property might be exploited to develop very sensitive immunoassays for human chorionic gonadotropin or any other antigen. Since the assays are not based on competitive inhibition between radiolabeled and unlabeled antigen, they are much more sensitive than a traditional radioimmunoassay in which either one of the same antibodies is used alone.*

The development of hybridoma technology facilitated preparation of antibodies that bind to a single antigenic determinant and thereby led to the development of valuable analytical reagents (*1*). Recent data show that combinations of selected monoclonal antibodies may be more useful for particular applications (*2–6*). We have shown that some mixtures of monoclonal antibodies to human chorionic gonadotropin (hCG) have a higher affinity than each individual antibody for the antigen (*2*). Since this effect was observed when $F(ab')_2$ fragments but not F(ab) fragments were substituted for the intact antibody, we concluded that the increase in affinity was a general phenomenon of bivalent antibodies, not unique to a peculiar feature of antibody-hCG interaction. Computer simulations of antibody-antigen binding suggested that the increase in affinity was caused by formation of a circular complex composed of two antigen molecules and one of each type of antibody (*7*). Biochemical evidence of a circular complex has also been obtained (*8*). The computer simulations also predicted that the binding of hCG to antibody mixtures would be cooperative and led us to perform the studies described here.

Monoclonal antibodies A102, B101, and B102 have been described previously (*2*, *9*). A102 binds the α subunit of hCG with an affinity for the intact hormone of 0.2 nM^{-1}. B101 and B102 bind the β subunit with affinities for the hormone of 0.7 and 0.03 nM^{-1}, respectively. Computer simulations in which we used the binding constants of A102 and B102 illustrate the binding of radiolabeled hCG as a function of hCG concentration and K', the relative probability of forming a circular complex (Fig. 1). The value of K' is similar to K_2 defined by Crothers and Metzger (*10*) for use in describing multivalent binding of antibodies to polyvalent antigens. The curves produced were biphasic and had an ascending limb at very low hCG concentrations. Estimates of the amount of radiolabeled antigen bound, based on ratios of bound to free hCG in Fig. 1A, can be obtained by using the relationship

$$\text{Radioactivity bound} = Cb/H_o = Cbf(1+bf)$$

where C is the total radioactivity (in counts per minute) added, b is the mass bound, f is the mass of tracer remaining free, and H_o is the sum of b and f. Use of this equation indicates that the amount of radiolabel bound increases approximately threefold over a wide range of K'. The shape of the curve is due to several components. At infinitesimal hCG concentrations, the ratio of bound to free hormone is equal to $2K_a[A102] + 2K_b[B102] + 4K_aK_b[A102][B102]$, where K_a is the equilibrium affinity constant of A102 for hCG and K_b is the affinity constant of B102 for hCG. As the concentration of hCG is increased from very low levels, the fraction of hormone bound as tetrameric circular complex increases. Since this is the most stable complex (*7*), the ratio of bound to free hormone increases. When approximately 50 to 75 percent of A102 is bound to hCG, it is nearly all in the form of the circular complex. Although the amount of complex increases further at higher hCG concentrations at the expense of free A102, the increase in amount of complex is less than that of total hCG. When this occurs, the ratio of bound to free hormone declines.

We prepared computer simulations using several different amounts of antibody, different antibody ratios, and antibodies with different affinities to learn which factors would increase the assay sensitivity. The sensitivity varied dramatically with the affinity of the antibodies for hCG. For example, a twofold increase in the affinity of each monoclonal antibody for hCG would result in a 32-fold increase in the sensitivity of a cooperative assay. Although, in principle, one can assay nearly any concentration of hCG with antibodies having only moderate affinity (as the case with A102 and B102), the ultimate limiting value for assay sensitivity depends on tracer-specific activity.

The cooperative immunoassay, which we designate CIA, can also be performed with radiolabeled antibody (Fig. 1B). In this example, we assumed that A102 was radiolabeled and that we had a second antibody specific for B102 and B102 complexes. Binding of radiolabeled A102 to B102 (measured as bound) would occur only through an hCG bridge. When K' was large, the maximum amount of A102 that could bind to B102 was equal to one half of the total hCG present (Fig. 1B, broken line). Although A102 would not bind to B102 through a circular complex when K' was zero, the binding that would occur through formation of linear complexes cannot be ignored (Fig. 1B, dotted line). The binding expected using experimentally observed values of K_a and K_b at several values of K' is illustrated by the solid lines. Since the circular complex composed of two molecules of hormone and one each of antibody accounts for greater than 90 percent of the bound hormone, measurements made with radiolabled A102 or radiolabeled hCG should have the same sensitivity. The position of the maximum in the binding curve is considerably different for both types of assay (compare A and B in Fig. 1). In the case when the anti-

body is radiolabeled, the decrease in binding is due to inhibition of circular complex formation by the hormone, an effect that occurs only at high antigen concentration, particularly when K' is large.

The prediction that small concentrations of unlabeled hCG would augment the binding of radiolabeled hCG to mixtures of A102 and B102 was confirmed experimentally (Fig. 2A). Higher concentrations of unlabeled antigen promoted a decrease in binding of radiolabel giving rise to a biphasic standard curve. The upward portion of the curve can be used to detect small concentrations of hCG. If multiple concentrations of unknown were assayed, both the ascending and descending limbs of the curve could be used to estimate hCG concentrations. To exclude the possibility that the cooperative effect would be observed for only those monoclonal antibodies that bound to individual subunits of hCG, we substituted B101, an antibody that binds to the β subunit of hCG, for A102 and observed a similar result (Fig. 2B). In the absence of B102, formation of the high-affinity complex is prevented, the positive slope is lost, and neither A102 nor B101 can be used to measure hCG concentrations less than 1 nM (not shown). Thus, the CIA based on antibody mixtures is at least 100-fold more sensitive than a displacement assay based on either antibody used separately. When used with monoclonal antibodies having slightly higher affinity, the CIA should be more sensitive than typical inhibition assays in which antisera with the highest possible affinity are used [K_a approximately 10^{12} (11)].

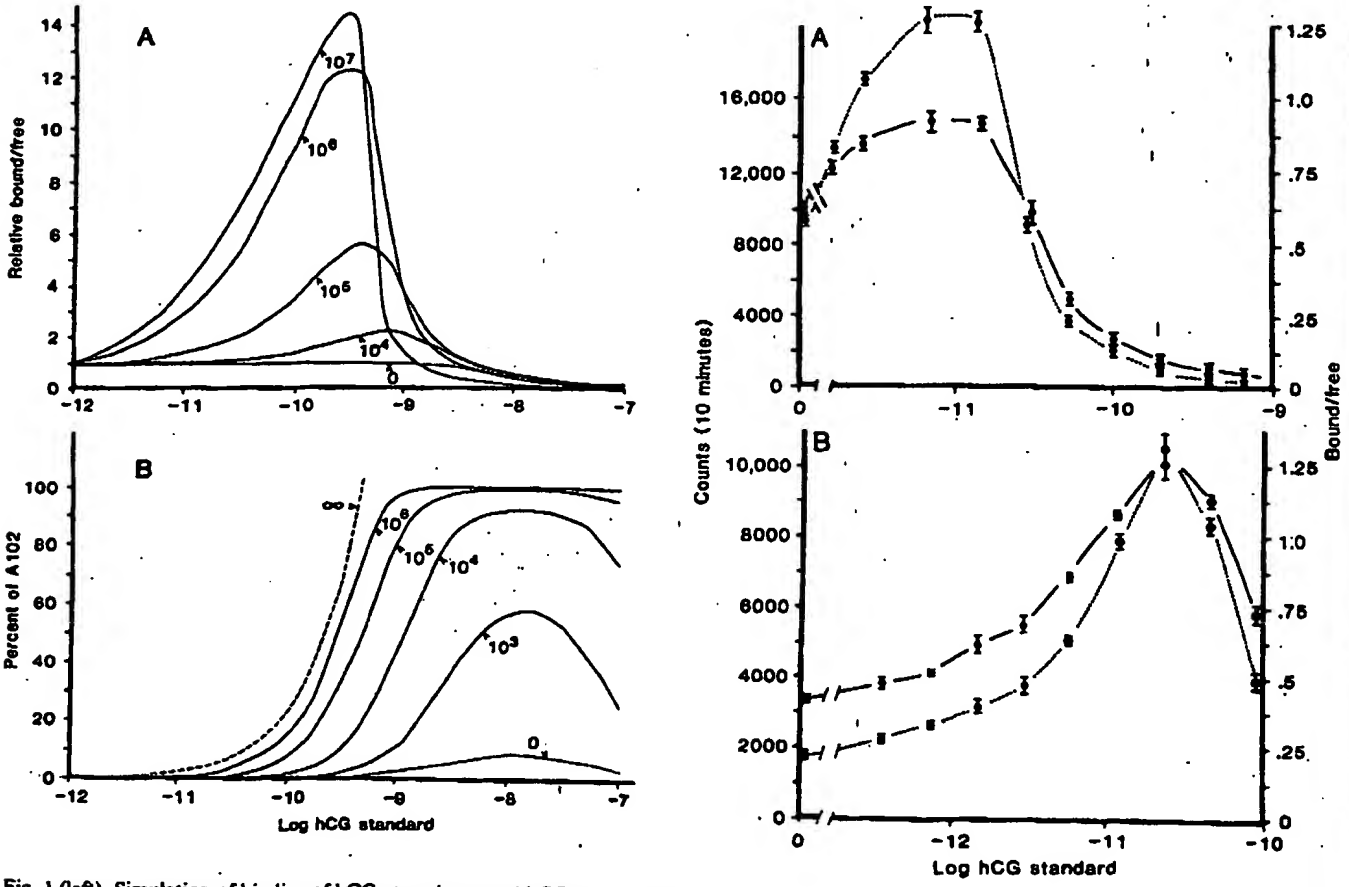

Fig. 1 (left). Simulation of binding of hCG at various total hCG concentrations. (A) Antibodies A102 (0.226 nM) and B102 (1.5 nM) were assumed to be mixed with radiolabeled hCG (1 pM), and various amounts of unlabeled hCG (1 to 10^5 pM) as shown in the abscissa. The ratio of bound to free hormone was calculated as described (*7*). Values graphed represent the ratio of bound to free hormone predicted in the presence of the unlabeled standard (that is, the concentration shown on the abscissa) divided by that ratio predicted in the absence of unlabeled hormone (that is, a constant value dependent on the concentrations of antibodies and antigen, the affinities of antibodies for the antigen, and the ability to form circular complex). Values of K' chosen for Fig. 1 (shown adjacent to each line) were within the range expected based on calculations made according to Crothers and Metzger (*10*). Although we do not have direct estimates of K', values of K' in excess of 100,000 would be sufficient to produce an observable cooperative interaction useful for CIA. (B) Conditions were identical to those in (A) except that A102 was radiolabeled. A theoretical maximal curve, shown furthest to the left, was calculated on the assumption that all the hCG added formed a circular complex involving A102 and B102. Values of K' (shown adjacent to each line) in excess of 1000 would be sufficient to observe cooperative interactions. The small amount of "cooperativity" seen when $K' = 0$ is due to interaction of the reagents to form the linear complex A102-hCG-B102. Fig. 2 (right). Standard curves for CIA's with mixtures of monoclonal antibodies. The methods used were the same as described previously (*2*, *9*) except that the amount of radiolabeled tracer in each assay tube was reduced at least fivefold. The total incubation volume was 0.3 ml. Bound and free hCG were separated by using antibody to mouse immunoglobulin as second antibody. Since only a small amount of radioactivity was used, the tubes containing the precipitate were counted for 10 minutes. The range of duplicate assays is indicated by the vertical lines. Solid line: raw counts per 10 minutes bound; broken line: calculated ratio of bound hCG to free hCG. (A) Results for a mixture of A102 (0.226 nM), B102 (1.5 nM), and radiolabeled hCG (1.6 pM). The total amount of radiolabel that could be bound by antibody was 27,000 counts per 10 minutes. (B) Results for a mixture of B101 (0.278 nM), B102 (1.34 nM), and radiolabeled hCG (1.6 pM). The total amount of radiolabel that could be bound by antibody was 17,700 counts per 10 minutes.

Immunoassays have had extensive plications in many fields. Although CIA's with the use of serum (*12–14*) have been described, the m chanism of the cooperativity has not been understood. On the basis of the observations of Weintraub *et al.* (*13*) that the effect is lost when F(ab) fragments are prepared, and on the basis of our findings with monoclonal antibodies (*2*, *7*), we assert that formation of a circular complex may frequently be the cause for cooperativity in antisera. Cooperative assays with antisera have had limited application because the range of cooperativity is small and factors needed to develop highly cooperative antisera are unknown. Assays based on monoclonal antibodies should hav more utility since appropriate ch ices of antibodies and their concentrations can be made to optimize the sensitivity and specificity of the assays. Use of multiple monoclonal antibodies also offers the possibility of increasing the specificity of the assay since both antibodies must bind simultaneously. Consequ ntly, as has been shown for a sandwich assay (*4*), the partial cross-reactivity of either antibody with interfering substances may be reduced in a factorial fashion. We have made similar observati ns for hCG (not shown). Requirements necessary to perform the CIA include antigens that have at least two epitopes, antibodies that bind to each epitope, and the ability of the antibodies and antigen to cooperate in binding. (We use the term "cooperativity" in the sense of forming a circular complex; cooperativity caused by an allosteric transition of the antigen is not required.) Practical application of the CIA with the use of radiolabeled antigen will require that all unknowns be analyzed at two concentrations because of the biphasic nature of the binding curve at low antigen concentration. Use of radiolabeled antibody should reduce this problem since the assays are biphasic only at very high antig n concentrations. As in all radioimmunoassays, the ultimate sensitivity of the assay is limited by the specific activity of the tracers employed. We envision that the CIA will be readily suited for nzyme immunoassays.

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Basement Membrane Collagen: Degradation by Migrating Endothelial Cells

Abstract. *One of the first steps in neovascularizaton is dissolution of the basement membrane at the point of endothelial outgrowth. An assay was developed to determine whether basement membrane collagens (types IV and V) are degraded by endothelial cells migrating toward a chemotactic stimulus. Fetal bovine endothelial cells were placed on one side of a filter containing the collagen substrate, and a chemoattractant derived from retinal extracts was placed on the opposite side. Degradation of both type IV and type V collagens was observed when the retinal factor was placed on the side of the filter opposite the endothelial cells. Metalloproteinases that cleaved type IV and type V collagens could be extracted from the endothelial cells with detergents. Such endothelial cell–associated (possibly membrane-bound) proteinases may locally disrupt the basement membrane and facilitate the outgrowth of capillary sprouts toward the angiogenic stimulus.*

Dissolution of the vascular basement membrane is associated with migration of endothelial cells out of the vascular channel toward an angiogenic stimulus (*1*, *2*). Individual migrating endothelial cells do not resynthesize a basement membrane until they become arranged in tubular capillary loops (*1*). The subendothelial basement membrane, a dense meshwork of collagen, glycoproteins, and proteoglycans (*3–7*), does not contain pores large enough to allow cell passage. Hence, movement of endothelial cells through the basement membrane may involve proteolytic disruption of this structure. Endothelial cells elaborate a collagenase that can degrade interstitial type I collagen (*8*), but this collagenase does not degrade type IV or type V collagens, which are the structural components of the basement membrane (*6*, *9*, *10*). Metalloproteinases that degrade type IV or type V collagens have been identified in tumor cells (*9*, *11*), macrophages (*12*), and bone (*13*) but not in endothelial cells. We therefore developed an assay to identify proteases capable of degrading basement membrane collagens.

A chemotactic stimulus was used to induce endothelial cells to migrate int a nitrocellulose filter containing bound, labeled, native type IV or type V collagen. Collagens labeled biosynthetically with [^{14}C]proline were purified as described (*9*, *11*, *14*), and 10 μg of the collagen dissolved in 0.05*M* tris-HCl, 0.9*M* NaCl, and 0.1 percent bovine thyroglobulin was incubated with nitrocellulose filters (Millipore SCWP; 13 mm; pore size, 8 μm) in a volume of 1 ml at 4°C for 10 hours. Substrate binding efficiency was greater than 90 percent. The filters were washed in phosphate-buffered saline, *pH* 7.4, and clamped in a chemotaxis chamber. The substrates were thus extracted, purified, and incubated with the nitrocellulose under nondenaturing conditions. The substrates were judged to be native because of their tertiary structure observed by rotary-shadowing electron microscopy and their insensitivity to digestion by α-thrombin at 30°C (*14*). The endothelial chemoattractant we used was derived from retina extracts (*15*). Fetal bovine endothelial cells (0.2×10^5) suspended in 1 ml of serum-free RPMI 1640 medium were applied to one side of the filter. After a 2-hour wait for the cells to attach, the retinal factor was added to one or both sides of the chamber. The chamber was incubated for 18 hours at 37°C. The filters were then removed, washed, and dissolved in scintillation medium (Biofluor); the amount of ^{14}C was determined in a liquid scintillation